ISSN 2320-5407 International Journal of Advanced Research (2014), Volume 2, Issue 5, 1169-1172

ISSN NO. 2320-5407

Journal homepage:http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Antifungal activity of *Cinnamomum verum* on Soybean seed-borne *Aspergillus flavus*.

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Manuscript Info

Manuscript History:

Received: 22 March 2014
Final Accepted: 25 April 2014
Published Online: May 2014

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Abstract

The aim of this study was to examine the efficacy of *Cinnamomum verum* J. Presl bark aqueous extract for its antifungal potential against soybean seed-borne *Aspergillus flavus*. *A. flavus* is dominant storage fungus colonizing soybean seeds due to its high nutrient composition. They produce aflatoxins which are toxic, immunosuppressive carcinogenic in nature and also causes seed rots, post-emergence damping off and reduces seed viability. At 500 mg/mL it showed complete inhibition of *A. flavus*. Thus the results obtained in this study demonstrate that *C. verum* bark aqueous extract possess a fungicidal activity which can be an alternative to synthetic fungicides for controlling soybean seed borne *A. flavus*.

INTRODUCTION

Soybean (*Glycine max* (L.) Merrill), is a major leguminous crop cultivated worldwide including India. Soy products are relatively inexpensive sources of high quality proteins, oil content which is in the form of food, feed and industrial uses (Sharma et al., 2014). USA, Brazil, and Argentina are the major producer of soybean seed in the world (Gupta et al., 2012). The world annual production of soybean is 254 million tons from an area of 102 m ha with an average yield of 2.49 tons per ha during the year 2010. Soybean seeds are infected with various seed-borne microorganisms, including fungi, bacteria and viruses (Krishnamurthy &Shashikala 2006; Sinclair 1982). The associated soybean seed-borne fungi *Aspergillus flavus*, *A. niger*, *Cercospora kikuchi*, *Macrophomina phaseolina*, *Fusarium oxysporum*, *F. solani*, and *F. moniliforme* etc., has been reported by many researchers (Krishnamurthy et al., 2008, Impullitti and Malvick, 2013). In Indian warm and humid storage condition, fungi are the most important microorganisms which causes seed deterioration resulting in loses of seed germination and viability (Shukla et al., 2009). *A. flavus* is one among the other fungi which colonizes the soybean seed during storage period (Bhattacharya and Raha, 2002). It causes seed deterioration by producing aflatoxins and causes pre- and post- emergence damping off seeds (Abdel et al., 2011). Aflatoxins has been classified as class one human carcinogen by the International Agency for Research on Cancer (IARC, 1993) is most potent carcinogenic, hepatotoxic, edema and immunosuppression.

Applications of synthetic fungicide has helped to reduce the soybean seed-borne fungi during storage period. The major problems with the constant use of synthetic fungicide is that resistance can be induced in fungi and increases the risk of high-level toxic residues to health and environment concerns (Carsten and Hjort, 2014). Thus, the use of biologically based compounds from plant extracts has been preferred as an alternative to fungicides to control phytopathogenic fungi. Plant products are cost effective and safe with no side effects. (Toyang and Verpoorte, 2013). Plants have been recognized long back to have a potential source of bioactive chemicals known as phytochemicals with potential antifungal agent and are biodegradable to non-toxic products (Dubey et al., 2010)

Materials and methods

Plant material

C. verum barks were purchased from local market of Bangalore, Karnataka, during January 2013. Dr. M. K. Sateesh confirmed the identification of the specimen. An authenticated voucher specimen of the plant is deposited in the herbarium of Molecular Diagnostic laboratory, Department of Microbiology and Biotechnology, Bangalore University, Bengaluru, Karnataka, India.

Preparation of aqueous plant extract.

C. verum crude aqueous bark extract was prepared according to Lakshmeesha et al., (2013). The *C. verum* bark material were rinsed in de-ionized water, oven dried at 35-40^{0}C for 48 hours and pulverized using a sterile electric blender to obtain a powdered form which was sieved and stored in sterile polyethylene sample bags prior to use. The aqueous extract of *C. verum* bark was prepared by soaking 10 g of powdered samples in 250 mL of sterile distilled water in 500 mL Erlenmeyer flask and boiled for 30 min. The supernatant was filtered through double layered muslin cloth and centrifuged at 8000 g for 15 min. It was filtered through Whatman No. 1 filter paper. The aqueous extract was subjected to rotary vacuum evaporator, frozen, lyophilized and dissolved in water at concentrations of 100 mg/mL to 500 mg/mL for antifungal activity.

Isolation of *A. flavus* from soybean seeds by agar plate method.

Soybean seed variety (JS-335) sample were collected during 2013 from GKVK, Bangaluru, Karnataka. The collected seeds were subjected to ISTA 1996 method. Soybean seeds were surface disinfected with 1 per-cent sodium hypochlorite solution for about 2 min at room temperature. Ten seeds per petri-dish were maintained and incubated in alternative cycles of dark and light for seven days at 27^{0}C on Potato Dextrose Agar (PDA) medium. After the incubation period, the petri-dish were observed for the seed-borne fungi under stereobinocular microscope, which revealed the occurrence of diverse fungal species. *A. flavus* (fig. 1) was selected for antifungal activities. *A. flavus* was identified based on their mycelial structure, growth, and spore morphology using standard manuals (Barnett and Hunter, 1972; Ellis, 1971; Sutton, 1971; Nagamani et al., 2006; Webster and Webber, 2007).

Antifungal investigations by poison food method

The required dose (100 mg/mL to 500 mg/mL) of the aqueous extract of *C. verum* bark was subjected to the poisoned food technique using Sabouraud Dextrose Agar (SDA) (Himedia Ltd, Mumbai) as nutrient medium. Requisite dose of the extracts (100 mg/mL, 200 mg/mL, 300 mg/mL, 400 mg/mL and 500 mg/mL) of the undiluted sample were mixed with the 20 mL of the SDA medium. SDA was aseptically transferred to pre-sterilized petri-dish. In to the medium 0.05 per cent Tween-80 was added for the even distribution of the extract. Once the medium had solidified, 5 mm disc of seven day-old culture of the *A. flavus* was inoculated aseptically to the center of the petri-dish. The plates were incubated at 27±2^{0}C for seven days. The SDA media devoid of the extract served as control. Captan was used as standard drug. Each test was replicated for three times and the fungi toxicity of the extract was measured after seven days in terms of per cent mycelial zone of inhibition. The percentage inhibition of mycelial growth was calculated by using the formula. Per cent inhibition = dc-dt/dc X 100

Where,

dc = average diameter of fungal colony in control petri dish

dt = average diameter of fungal colony in treated petri dish

Results

Soybean seed (JS 335) cultivar when subjected to agar seed health test revealed the presence of *A. flavus* along with other fungi. Identification of the fungus was done by colony morphology and the light microscopic properties. The activity of the plant extracts against the mycelium growth of *A. flavus* is presented in Table 1. It was observed that out of five concentrations, 500mg/mL showed complete inhibitory effect against the mycelium growth of *A. flavus* when compared to negative control (Fig. 2). The 400 mg/mL, 300 mg/mL, 200 mg/mL and 100 mg/ mL showed 69.9 ± 7, 62.59 ± 5, 61.34 ± 3 and 55.21 ± 03 respectively showed moderate inhibitory effect against the mycelium growth of *A. flavus*.

Fig. 1. Soybean seed showing heavy colonization by *A. flavus*. ISTA method (after 7 days)

Table 1. Effect of *C. verum* aqueous extract on mycelial growth inhibition of *A. flavus*

	A. flavus						
Concentration mg/mL per cent of Inhibition	**100**	**200**	**300**	**400**	**500**	**Negative control**	**Positive control (50 µg/mL)**
	55.21 ± 1.1	61.34 ± 17	69.59 ± 0.8	89.9 ± 1.2	100	0	100

The above-mentioned readings are exclusive of the 5mm diameter. Observations are expressed as mean ± standard error, (n=3).

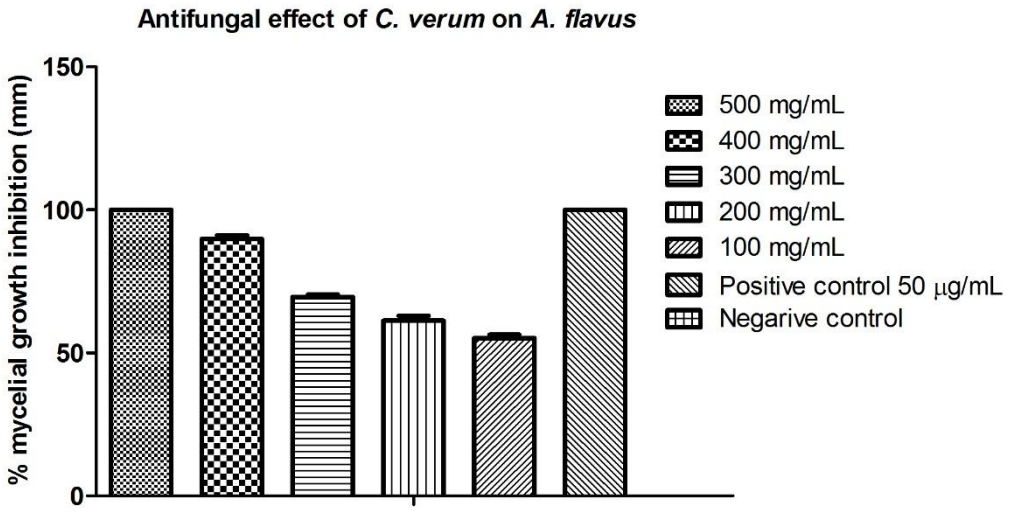

Fig. 2. Antifungal activity of *C. verum* barks extracts against the soybean seed-borne fungus *A. flavus*.

Discussion

A. flavus was found to be seed-borne fungi in soybean seeds. Krishnamurthy et al.,(2008) reported that *A. flavus* isolated from soybean seed sample will degrade the seed nutrients during storage period. The present *in vitro* study has demonstrated valuable data, indicating of the differential activities of the *C. verum* bark aqueous extracts on the mycelium growth of *A. flavus*. Similar antifungal studies were carried out by Ahmed and Hussein (2012),Dabur et al. (2004), and Singh et al. (2007) using botanicals extracts to inhibit the growth of *A. flavus*. However, the fungi A. flavus used in this study was an isolate of soybean seeds.

The results presented in this study provide valuable data, *C. verum* aqueous extracts may assist in the registration and development of a fungicide against *A. flavus*. Therefore further studies should be conducted to purify an active compounds of *C. verum* bark which may yield significant fungicide against *A. flavus*.

Acknowledgements

The authors convey sincere thanks to UGC for providing financial support from the RGNF and Dept. of Microbiology and Biotechnology, Bangalore University for providing lab facilities.

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